



Letrozole-loaded nonionic surfactant vesicles prepared via a slurry-based proniosome technology: Formulation development and characterization

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ABSTRACT

Slurry-based Letrozole (LTZ)-loaded proniosomes were designed using sucrose or sorbitol as carriers and various ratios of cholesterol (CH) and Tween 80 (T80) as lipid composition. Proniosomes were hydrated and probe-sonicated to generate nano-vesicles. The proniosome powders were characterized in terms of morphology using scanning electron microscopy, and drug crystallinity using differential scanning calorimetry (DSC) and X-ray diffraction (XRD). The niosomes generated from proniosomes were characterized and compared to conventional niosomes, in terms of size, zeta potential, drug entrapment, storage stability, and drug release. All formulations had size measurements in the range of 100–194 nm, polydispersity index (PDI) values below 0.3, and zeta potential values below –23 mV. Drug entrapment was the highest for niosomes generated from sucrose-based proniosomes (CH:T80; 1:1), reaching 74% compared to less than 50% for conventional niosomes. Storage for 3 months at 4 °C resulted in minor drug leakage whilst most drug was leaked from vesicles stored at room temperature. DSC and XRD studies showed that LTZ was converted into its amorphous form upon incorporation into proniosomes. Drug release exhibited a biphasic pattern, being fast at the first 24 h (up to 65% released) followed by a very slow release phase for a duration of one month, releasing at least 95%. The release profile of niosomes fits best with the Higuchi model. Overall, in this study, a facile approach to generating niosomes incorporating LTZ using a slurry-based proniosome technology was demonstrated. The niosomes provided high drug entrapment and controlled biphasic release over one month.

1. Introduction

Breast cancer is a leading global cause of cancer-related mortality in women [1,2] and is becoming increasingly common amongst female patients below 40 years old [3,4]. In the Gulf Cooperation Council (GCC) countries (the region where this research paper has been originated), breast cancer represents about 23% of all female cancer cases between 1998 and 2007, and the percentage is expected to increase in 2020 [5].

Amongst many anticancer agents, letrozole (LTZ), a third-generation selective non-steroidal aromatase inhibitor, has obtained FDA approval as an adjuvant treatment of estrogen-positive breast cancer in postmenopausal women [6,7]. Femara® (2.5 mg), amongst other similar formulations, is a clinically established oral tablet product of LTZ. The

drug is given in a low oral dose because of its adverse effects, including arthralgia, bone fragility, and hypercholesterolemia [8], with recent reports indicating that hormone fluctuations due to administration of LTZ may cause mental depression [9]. The large volume of distribution of LTZ is responsible for the low plasma levels of the drug, and the numerous adverse effects, which may compromise the overall therapeutic benefit [10].

Developing prolonged release parenteral infusions or intratumoral injections of LTZ can be highly advantageous for minimizing the potential of adverse effects and increasing the localization of the drug at the cancer site. However, the design of such alternative formulations of LTZ is challenged by the poor aqueous solubility of the drug, and the shortage of information related to its stability in aqueous dosage forms.

Biodegradable drug delivery systems, including nanotechnology-

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based formulations, have attracted considerable attention over the past few decades. These delivery systems can overcome poor solubility, enhance therapeutic efficacy, and reduce the adverse effects of a wide variety of therapeutic molecules [11–14]. Recent development of LTZ formulations utilizing polymeric systems has been reported, through employing chitosan nanoparticles [15], chitosan-lipid nanocomplexes [16], dendrimers [17,18], and other polymers like poly(D,L-lactide) (PDLLA) to prepare microparticles [19] or nanoparticles [20]. In our laboratory, we adapted an aerosol generating mechanism to produce monodisperse biodegradable polycaprolactone (PCL) and PDLLA LTZ particles. The resultant formulations exhibited superior drug entrapment efficiencies (94–97%), gradual drug release over one month, and in vitro cytotoxicity against the cancerous breast cells MCF-7 [19].

As an alternative to polymers, surfactants such as phospholipids (e.g., naturally derived phosphatidylcholines) or synthetic non-ionic surfactants (e.g., Tweens and Spans) have been used to design liposome or niosome formulations, respectively. Amongst many types of nano-carrier delivery systems, liposomes have been reported for the earliest success in anticancer therapy, and several liposome-based anticancer formulations have been commercialized [21,22]. For example, Doxil® is an intravenous injection of PEGylated liposomes loaded with doxorubicin; this formulation proved to be efficacious in the management of solid tumours, haematological malignancies and AIDS-related Kaposi's sarcoma [23,24].

Unfortunately, liposomes are unstable carriers, because of liability of their phospholipid molecules to hydrolysis and oxidation, with concomitant vesicle aggregation and leakage of the originally entrapped drug. Niosomes (non-ionic surfactant vesicles) are stable alternatives to liposomes [25,26]. Azizi and Norouzi (2015) have developed a PEGylated niosomal formulation of LTZ, and reported that the niosomal formulation was toxic to MCF-7 cancerous cells in vitro [27]. Thin film hydration method is one of the most extensively studied conventional techniques and has been reported to generate multilamellar niosomes [28]. Unfortunately, liposomal and niosomal formulations prepared using such conventional techniques (e.g. thin film hydration) are difficult to scale up and may demonstrate instability manifestations during their storage as aqueous dispersions.

Proniosomes are stable dry powder precursors of niosomes, offering higher stability profile and greater potential for large scale manufacturing. Proniosomes are carbohydrate particles coated with non-ionic surfactants that can generate niosome vesicles upon addition of aqueous phase with shaking [29–32]. Scaling up studies of proniosomes have been conducted by compressing the proniosome powders into tablets [33], or by the large-scale generation of nano-niosomes from proniosomes via hydration followed by high pressure homogenization [34]. Niosomes generated using proniosome technology may offer potential for delivery via a range of routes such as oral [33,35,36], parenteral [37], and pulmonary [30].

In the present study, we designed LTZ-loaded proniosome formulations that can readily be hydrated into niosomes and reduced to the nano size via probe-sonication. The nano-niosomes were thoroughly characterized in terms of particle size and size distribution, zeta potential, and ability to entrap LTZ and sustain its release. The profile of drug release was studied over a prolonged period of one month, and the release mechanism was identified and described by considering the following models: zero-order, first order, Higuchi, Korsmeyer-Peppas, Weibull, Hill equation and Hixon Crowel [38]. Furthermore, the proniosome powder formulations were characterized in terms of proniosome surface morphology, and drug content and crystallinity. To the best of our knowledge, this is the first study that employed a slurry-based proniosome technology to incorporate LTZ in controlled release formulations. It is hypothesized in this study that niosomes generated with this method would have potential, subject to further investigations, to be used as an intratumoral injection, with the aim of providing an alternative to the commercially available Femara® oral tablets.

2. Materials and methods

2.1. Materials

Cholesterol (CH), carbohydrate carriers (sorbitol and sucrose), Tween® 80 (T80; polysorbate 80; polyoxyethylene sorbitan mono-oleate) and dialysis bags (cut-off 12,000 Da) were all purchased from Sigma-Aldrich, Germany. Chloroform and acetonitrile were both of HPLC-grade and purchased from Merck Co., Germany. Transcutol® HP was supplied by Gattefossé, Lyon, France. Letrozole (LTZ) was bought from Jiangsu Ainty Handsome CO., LTD, China. All materials were used as received without further modification.

2.2. Methods

2.2.1. Preparation of niosomes using thin film hydration

Tween® 80 (T80) and other lipid phase components such as cholesterol (CH) and letrozole (LTZ) were mixed and dissolved together in chloroform (10 ml) within a round bottomed flask (25 mg/ml). The flask was attached to a rotary evaporator (Buchi Rotavapor, Switzerland) for 2 h to allow the organic solvent to evaporate under reduced pressure and leave a thin lipid film on the inner wall of the flask [28]. The flask was left under fume hood overnight to allow for evaporation of solvent residues, if any. The film was then hydrated with deionized water (60 °C) followed by mechanical shaking (Julabo® shaking water bath, USA) for 30 min to ensure complete lipid hydration. The formulation was left on the bench overnight before performing further processing or characterization. The composition of all formulations is elucidated in Table 1.

2.2.2. Preparation of niosomes using the slurry-based proniosome technology

Proniosomes were prepared by adapting the slurry-based proliposome method we previously introduced [39]. Sucrose or sorbitol carrier particles were sieved using a sieving set (Fisher Scientific, UK) to obtain a size range of 250–500 µm. Proniosomes were prepared by coating T80 and CH (250 mg total lipid phase), in various molar ratios (Table 1) onto sucrose or sorbitol carrier particles, in 1:5 w/w lipid to carrier and by incorporating LTZ into the lipid phase (5% or 30% of the lipid phase). This was achieved by loading the carbohydrate carrier particles (sucrose or sorbitol) into a round bottomed flask. T80, CH and LTZ were dissolved in ethanol separately and poured directly on the carbohydrates carriers to create a slurry. The flask containing the alcoholic slurry (333 mg/ml) was attached to the rotary evaporator and partially immersed into a water bath (45 °C), allowing ethanol to evaporate under rotation and reduced pressure for 2 h. The flask was placed under the fume hood overnight. The proniosomes were collected from the flask and stored in glass vials at 4 °C for subsequent processing and characterization. The composition of all proniosome formulations is

Table 1
Different formulations for niosomes and proniosomes.

Formulation	CH:T80	Carrier	Loaded with LTZ	Hydration Temperature (°C)
F1	1:1	N/A	Yes	60
F1B	1:1	N/A	No	60
F2	1:0.5	Sorbitol	Yes	60
F3	1:0.5	Sucrose	Yes	60
F4	1:1	Sorbitol	Yes	60
F4B	1:1	Sorbitol	No	60
F5	1:1	Sucrose	Yes	60
F5B	1:1	Sucrose	No	60
F6	1:1.5	Sorbitol	Yes	60
F7	1:1.5	Sucrose	Yes	60
F8	1:2	Sorbitol	Yes	60
F9	1:2	Sucrose	Yes	60

shown in Table 1. Niosomes were generated from proniosome granules (containing 1.5 mg LTZ) by placing the granules in an Eppendorf tube and adding deionized water (1 ml; 65 °C) with vortex-mixing for 2 min. The tubes were then placed in a *Julabo*® shaking water bath set at 60 °C for 30 min. The formulations were left at room temperature overnight to allow for niosome stabilization.

2.2.3. Vesicle size reduction via sonication

Following the generation of niosomes from thin films or proniosomes, the tubes containing niosomes were placed in a Branson® water bath sonicator (Branson Ultrasonics, Connecticut, USA) for 30 min at room temperature to facilitate deaggregation of aggregated vesicles. This was followed by probe-sonication (Branson® probe sonicator, Branson Ultrasonics, Connecticut, USA) for 6 min, and by allowing for intermittent cooling, every 30 s for 2 min to avoid overheating of the formulations above 70 °C. The samples were spun for 10 min to sediment the titanium particles leaching from the probe of the sonicator. The samples were then aspirated from the supernatant for subsequent characterization studies.

2.2.4. Particle size analysis and zeta potential studies of niosomes

The hydrodynamic diameter and size distribution (polydispersity index; PDI) of all niosome formulations were analyzed via dynamic light scattering using the Nano ZS Zeta Sizer instrument (Malvern Instruments Ltd, UK) at 25 °C. For each sample, three independent measurements using three different batches were performed. Zeta potential analysis was conducted using Laser Doppler Velocimetry by employing the same instrument at 25 °C following selection of the relevant software option.

2.2.5. Drug entrapment determination using Ultraperformance Liquid Chromatography (UPLC)

Reliable determination of drug entrapment depends on adequate separation of the entrapped drug from the untrapped fraction. In other words, one obstacle in drug entrapment determination is the concomitant sedimentation of the vesicles (containing the entrapped drug) with the untrapped free drug particles upon centrifugation. This commonly results in significantly overestimated drug entrapment efficiencies [39]. In one of our earlier studies, the employment of deuterium oxide (i.e. heavy water) has demonstrated to be an appropriate dispersion medium to separate vesicles from the untrapped fractions of another lipophilic drug upon centrifugation [39]; therefore, the utilization of deuterium oxide to better separate LTZ in our experiments was also investigated. Two separation methods were used before the determination of LTZ entrapment efficiency in the niosomes, by using two different dispersion media. The separation of entrapped drug from the untrapped (free) fraction in this study was adapted from the procedure we previously published with liposomes entrapping beclometasone dipropionate [39]. One of the two methods is the separation from vesicles dispersed in deionized water (DW) followed by centrifugation; thus, vesicles (with entrapped drug) would presumably sediment whilst untrapped drug remains in the supernatant (Fig. 1A and B). In fact, this method has shown to provide an overly estimated entrapment in liposomes due to the concomitant sedimentation of liposome vesicles (incorporating the drug) and the free (untrapped) insoluble drug crystals [39].

The second method, which has shown to be more accurate in our previous studies, was implemented by hydrating the proniosomes with deuterium oxide (i.e. D₂O; heavy water), followed by centrifugation at optimum conditions (Fig. 1A, C). The use of the higher density medium (i.e. D₂O) may facilitate the separation of niosomes so that, upon centrifugation, vesicles tend to float on the surface where they can be aspirated to quantify the entrapped hydrophobic drug. According to our previous findings using this method, the untrapped drug is sedimented in the form of solid particles that have a higher density than the lipid vesicles [39]. The difference between the two separation methods

is illustrated in Fig. 1. Thus, for each formulation, two sets of samples were hydrated using deionized water (DW) or heavy water (D₂O). Both sets of samples were sonicated as described earlier, and then placed in the Thermo Scientific® Micro 21R centrifuge (Thermoscientific, USA), for 45 min at 15,500 × g at 4 °C, to separate the niosomes (including a proportion of LTZ) from the free (untrapped) drug. The separation in case of D₂O following centrifugation was ascertained using light microscopy by viewing samples from the floating creamy layer, the sediment, and the continuous phase in between the two regions.

For the deuterated water (D₂O) method, the cloudy floating layer (100 µl) was carefully aspirated and further diluted with acetonitrile (1.9 ml) in order to disrupt the vesicles and solubilize the entrapped LTZ. The resultant solution samples were vortex-mixed for 2 min and filtered through Polytetrafluoroethylene (PTFE; 0.2 µm; Sigma Aldrich, Germany) before loading into Waters® Ultraperformance Liquid Chromatography (UPLC) vials for subsequent drug quantification using UPLC (Waters, Illinois, USA) at 240 nm by following a method we recently published [20].

- For the first method, using deionized water (DW) as a dispersion medium:

$$EE(\%) = \frac{\text{Total drug amount in the sample} - \text{Drug amount in the supernatant}}{\text{Total drug amount in the sample}} \times 100 \quad (1)$$

- For the second method, using heavy water (D₂O) as dispersion medium:

$$EE(\%) = \frac{\text{Drug amount in the floating top layer (i. e. niosome layer)}}{\text{Total drug amount in the sample}} \times 100 \quad (2)$$

2.2.6. X-ray diffraction studies

The X-ray Diffraction (XRD) analysis of proniosome powders (manufactured using the slurry method) and LTZ raw powder was conducted at room temperature by using the X-ray diffractometer D8 Advance with CuK alpha radiation source (Bruker Co., Germany). The diffraction data were collected over the angular range of 5–60° with a step size of 0.05° and a counting time of 60 ms. The other various components were assigned through auto-fitting in the instrument using the DIFFRAC.EVA software (Bruker Co., Germany). In this experiment, LTZ-loaded proniosomes were prepared with a drug concentration of up to 50% (w/w) of the total lipid, taking into account that the concentration of LTZ used in the formulations might be below the limit that can be detected by the instrument. Furthermore, LTZ alone was processed in the rotary evaporator with chloroform to end of up with a dry product, for subsequent comparison with the crystallinity of LTZ incorporated into the proniosome powders.

2.2.7. Differential scanning calorimetry (DSC)

Thermal properties of the proniosomes powders were studied using differential scanning calorimetry (DSC) by employing the DSC 8000 instrument equipped with an intra-cooling system Intra-cooler II (PerkinElmer, USA). All samples were analyzed at a heating rate of 10 °C/min in the range of 30–220 °C.

2.2.8. Scanning electron microscopy (SEM)

Proniosome powder samples were visualized using scanning electron microscopy (SEM; JSm 6100 JEOL, Tokyo, Japan). The samples were mounted onto the SEM stub and coated with gold film (200 nm thickness). The experiments were performed under reduced pressure (0.001 mmHg) and images were taken to evaluate the morphology of the proniosome particles.

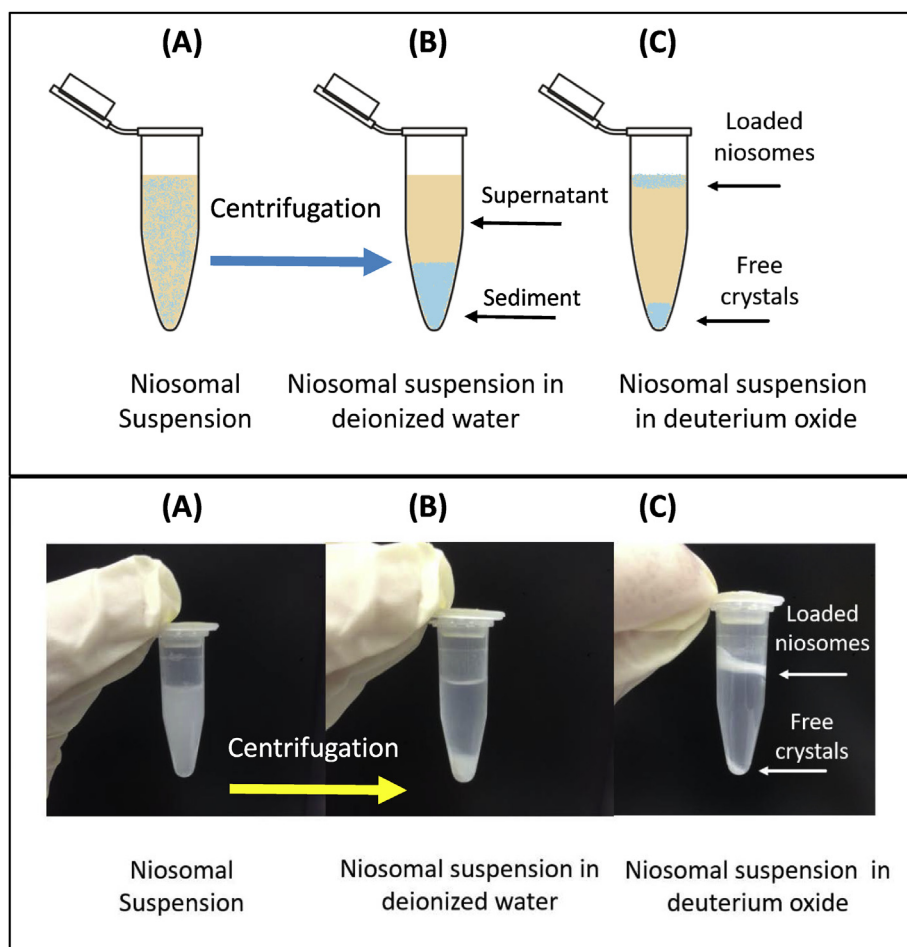


Fig. 1. Niosomes separation using deionized water (DW; H₂O) or deuterated water (D₂O) as hydration media. (A) The dispersion prior to centrifugation where niosomes were hydrated and are uniformly distributed in the suspension. (B) Following centrifugation of the dispersion in DW, niosomes (containing entrapped drug) and the free (unentrapped) LTZ crystals are sedimented at the bottom of the tube. (C) Three distinctive layers are present upon centrifugation of the dispersion in D₂O; the top floating layer represents the niosomes (with entrapped drug) while the free crystals are sedimented. Trace fraction of the drug in its water-soluble form is associated with the clear aqueous phase in the middle region.

2.2.9. Assessment of drug release from niosomes in vitro

Niosome formulations were evaluated for the release rate of LTZ using the membrane diffusion technique. Dialysis bags (cut-off 12,000Da, Sigma, Germany) were used as 'donor compartment' by incubation for 24 h in a phosphate buffer (containing 20% Transcutol as co-solvent) to maintain a sink condition, at a physiological pH of 6.8 [40]. The niosomes containing LTZ were pipetted into the dialysis bags, which were immersed in phosphate buffer to end up with a final volume of 100 ml. The bags were kept in a shaking water bath (Julabo®, USA) for one month at 37 °C ± 1 and 100 rpm. A comparative sample consisting of LTZ suspension was prepared by weighing an equivalent amount of LTZ and mixing it in phosphate buffer containing 20% Transcutol. The mixture was vortexed for 10 min and then placed in a dialysis bag and clipped before placing it in the recipient medium.

At time intervals, 3 ml of the dissolution medium was taken and replaced with an equal volume of fresh drug-free medium. The samples were separately measured using UPLC, and results were presented as mean values of three different runs using three different niosome batches. The release data were fitted to a range of models, which were zero-order, first order, Higuchi, Korsmeyer-Peppas, Weibull, Hill equation, and Hixon Crowell [38], in order to determine the mechanism of drug release. The correlation coefficients for the different release profiles were also determined. All kinetic profiling studies were conducted using KinetDS3 software, where the r^2 values were calculated by regression analysis of each model [38].

2.2.10. Statistical analysis

The results were reported as mean ± standard deviation for three measurements conducted on three different batches. The difference between the groups was regarded to be significant when P values were

equal to or smaller than 0.05. These measurements were conducted using the Sigma plot software (version 12.0) by performing the *t*-tests and one-way analysis of variance (ANOVA) for two groups and more than two groups, respectively.

3. Results and discussion

3.1. Size of niosomes

Table 1 represents all formulations investigated for size analysis including those made by the conventional thin-film hydration method (F1 and F1B), and niosomes made using the proniosome technology (F2-F9). As shown in Table 2, the effect of drug inclusion on the measured size and polydispersity was investigated for thin-film niosomes (F1 and F1B) and for niosomes generated from proniosomes (F4 and F4B, F5 and F5B). The effect of LTZ inclusion in the formulations was studied for both carriers (sorbitol and sucrose) but only for CH:T80 (1:1) (Table 2) because this ratio is commonly used in published reports, due to established formulation stability and minimized drug

Table 2
Size analysis of LTZ-loaded and unloaded niosome formulations.

F#	Carrier	Particle size (nm)	PDI
F1B	N/A	60.4 ± 1.6	0.20 ± 0.008
F1	N/A	120.3 ± 21.5	0.20 ± 0.035
F4B	Sorbitol	85.9 ± 0.6	0.14 ± 0.010
F4	Sorbitol	148.4 ± 9.8	0.16 ± 0.021
F5B	Sucrose	93.5 ± 5.2	0.16 ± 0.013
F5	Sucrose	139.2 ± 2.5	0.17 ± 0.057

Table 3
Size analysis, polydispersity index, and zeta potential for all formulations.

F#	CH:T80	Carrier	Particle size (nm)	PDI
F1	1:1	N/A	120.3 ± 21.5	0.197 ± 0.035
F2	1:0.5	Sorbitol	194.3 ± 3.8	0.268 ± 0.008
F3	1:0.5	Sucrose	146.8 ± 29	0.185 ± 0.048
F4	1:1	Sorbitol	148.4 ± 9.8	0.159 ± 0.021
F5	1:1	Sucrose	106.8 ± 13.3	0.168 ± 0.056
F6	1:1.5	Sorbitol	117.4 ± 5.3	0.283 ± 0.031
F7	1:1.5	Sucrose	105.1 ± 4.2	0.197 ± 0.036
F8	1:2	Sorbitol	102.6 ± 3.2	0.270 ± 0.028
F9	1:2	Sucrose	100.3 ± 6.3	0.250 ± 0.033

Values expressed as Mean ± SD (n = 3).

leakage [35,41–45]. Inclusion of LTZ caused the hydrodynamic size to increase significantly ($p < 0.05$) with only a slight trend of increment in PDI (Table 2). The increase in hydrodynamic size as a result of drug incorporation was most prominent for vesicles prepared using thin-film hydration (F1B and F1), so that size was doubled (Table 2). By contrast, the increase in particle size, caused by drug inclusion, was least when vesicles were generated from proniosomes using sucrose carrier (F5B and F5; Table 2). The increase in niosome size as a result of drug inclusion has previously been reported [46–48]. Size increment might be attributed to an increase in the hydrophobicity of particle surfaces, promoting aggregation and/or fusion of the individual particles [30]. It has also been hypothesized that materials incorporated into surfactant vesicles may interact with the polar headgroups of the surfactant, causing repulsion of the surfactant bilayers and increasing vesicle size [46,47].

Table 3 demonstrates a comparison between all formulations that included LTZ, hence, the effect of carrier type and lipid phase composition of the niosomes was studied. Generally, both hydrodynamic size and PDI were affected significantly ($p < 0.05$) by the changes on each formulation. Overall, Table 3 demonstrate that particle size of the generated niosomes using the conventional or proniosome-based techniques ranged between 100 and 194 nm. Furthermore, the vesicles generated from proniosomes were similar in size to those made using the conventional thin-film method ($p > 0.05$). In addition, regardless of carrier type, the increment of CH proportion in the formulation caused an increase in particle size (Table 3), possibly through increasing the hydrophobicity of the particles, promoting aggregation, as described earlier.

The size distributions, expressed as PDI, were below 0.3 (Table 3), indicating that niosomes had low polydispersity, regardless of formulation composition [44]. Niosomes had the lowest PDI when CH:T80 was 1:1, suggesting that the equimolar ratio is most appropriate for having vesicles with narrow size distribution (Table 3). Previous reports demonstrated that maximum niosome stability is achieved when CH and surfactants are incorporated in 1:1 mol ratio [48]. In general, niosomes generated from sucrose-based proniosomes showed similar or lower PDI when compared with vesicles generated from sorbitol-based proniosomes.

The particle size measurements reported in the current study come in agreement with previous literature reports. The mean diameter reported for niosomes prepared using T80 and CH followed by sonication was below 300 nm, with a PDI of 0.49 [49]. Additionally, T80 niosomes were found to exhibit smaller particle size when compared to other Tween surfactants [50], justifying the use of T80 in the present study.

3.2. Zeta potential analysis

Zeta potential can be used as an indicator of the extent of repulsive forces between the dispersed nanoparticles [42]. In general, zeta potential of niosomes was found to be negative for all formulations in a manner that was dependent on formulation (Fig. 2A). The zeta

potential, in terms of charge intensity, of all niosomes was found to be less than -30 mV (Fig. 2A). Niosomes generated from sucrose-based proniosomes had equal or lower zeta potential values than vesicles generated from sorbitol-based formulations (Fig. 2A). To study the influence of LTZ incorporation on zeta potential, we used CH:T80 (1:1) as the standard lipid composition for thin-film made formulation and proniosome-generated vesicles (sucrose-based and sorbitol-based) (Fig. 2B). Our investigation indicated that LTZ had a trend of intensifying the negativity of surface charge for proniosome-made formulations; this, however, was statistically insignificant ($P > 0.05$). However, for thin-film made niosomes, the effect of LTZ at increasing the charge intensity was tremendous ($P < 0.05$), because the drug-free vesicles were found to have a very mild negative zeta potential (Fig. 2B).

3.3. Drug entrapment efficiency in niosomes

Drug entrapment was determined following separation of untrapped drug from that entrapped in the vesicles. Separation can be achieved using numerous techniques. A traditional technique commonly reported in literature is implemented by centrifuging the vesicle dispersion in order to sediment the vesicles (with the drug entrapped) and leaving an aqueous supernatant where the untrapped drug is presumably located [42,43,49,51]. Although this technique is widely and commonly used, research investigations have demonstrated it to be inaccurate when the incorporated drug is hydrophobic [39,52,53]. In aqueous phase, hydrophobic drugs tend to form crystals with density similar to that of vesicles, resulting in concomitant sedimentation of the untrapped drug crystals with the vesicles containing the entrapped drug upon centrifugation [52]. Thus, the sedimented crystals are falsely counted as entrapped drug, resulting in overestimated drug entrapment in vesicles [39]. We resolved this problem and optimized the separation in our earlier studies (using liposomes as model vesicles) by dispersing the liposomes in deuterium oxide (heavy water; D_2O) instead of water. Centrifugation in D_2O using optimum conditions (i.e. temperature, speed and duration) causes preferential localization of the vesicles on the surface of dispersion as a floating creamy layer, whilst solid drug particles tend to sediment as demonstrated using light microscopy (Fig. 3). Furthermore, centrifugation conditions depend highly on type and density of drug crystals/solid particles [53]; thus, the centrifugation conditions apply here for LTZ loaded into niosomes may not apply on other hydrophobic drugs and different vesicle compositions. It is important to bear in mind that the use of D_2O was confined to the entrapment studies; this solvent is not part of the preparation protocol owing to its dose-related toxicity [54] and high cost.

In the present study, we reported the entrapment efficiency of LTZ as ‘actual entrapment’ using D_2O , and for comparison, we also determined the ‘apparent entrapment’ using DW as dispersion medium (Fig. 2A). LTZ was initially incorporated into the formulations in excess (30% w/w) aiming to achieve maximum entrapment, and that was compared with 5% w/w loading. The ‘apparent entrapment’ of LTZ was similar for both loadings whilst the ‘actual entrapment’ tended to be either similar for both loadings, or different between the two loadings, but with no clear trend or rule (Fig. 4A). Noteworthy, in most formulations, the ‘actual entrapment’ was higher for the higher loading (30%) compared to the lower loading (5%) (Fig. 4A). This is contrary to our expectation since entrapment is calculated here as ‘entrapment efficiency’ (i.e. the percentage proportion of entrapped drug) which is expected to be higher when the loaded drug amount is lower. The unexpected finding might be attributed to the presence of some LTZ in its water-soluble form in the aqueous region between the floating creamy layer (i.e. niosome layer) and the drug sediment at the bottom (Fig. 3). This water-soluble fraction of LTZ was a significant proportion when the lower drug loading (i.e. 5%) was used.

Fig. 4A also demonstrates that the apparent drug entrapment efficiencies were significantly higher ($P < 0.5$) than the actual

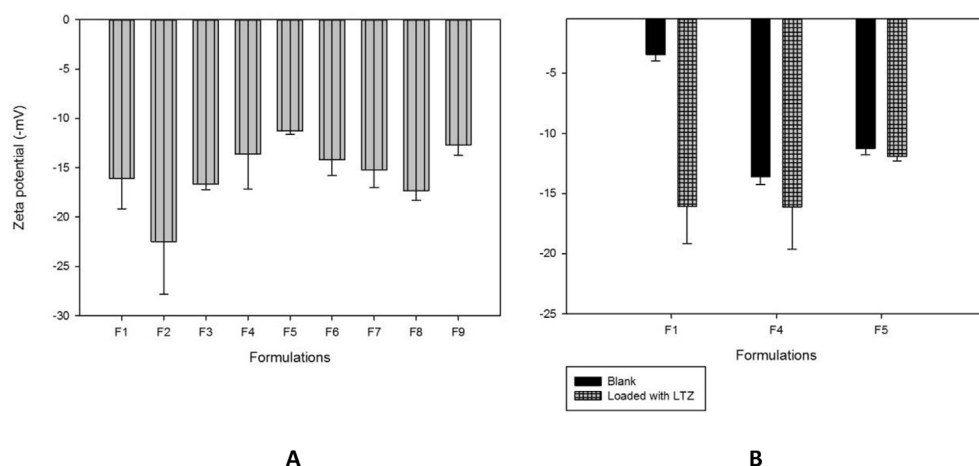


Fig. 2. A. Zeta potential difference between blank (i.e. drug-free) niosomes and LTZ-loaded niosomes. Error bars represent standard deviation from three independent experiments using three different proniosome batches. B. Zeta potential for all formulations. Error bars represent standard deviation from three independent experiments using three different proniosome batches.

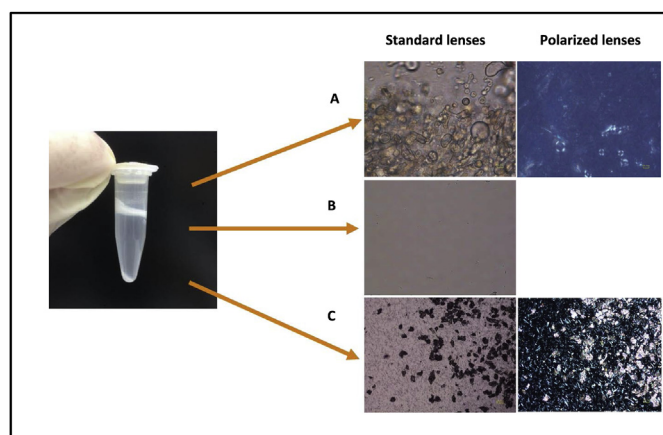


Fig. 3. Optical microscopy images for the three distinctive layers obtained by using heavy water (D₂O) as the hydration medium for the niosomes. Optical microscopy images were evaluated under standard and polarized lenses (left and right images, respectively). A. The top images are for the floating creamy layer, representing a confirmation for the generation of the vesicles with minimal to no crystals deposition out of the niosomes. B. The middle image is for the continuous aqueous phase where it contains no vesicles or crystals, but a trace fraction of soluble LTZ. C. The bottom image is for the sedimented untrapped crystals of LTZ.

entrapment efficiencies. The maximum actual entrapment was achieved upon using a surfactant to cholesterol ratio of 1:1 and 1.5:1, regardless of carrier type. The least entrapment was reported to be in formulations F2 and F3, in which the ratio of CH to surfactant was 1:0.5 (Table 1), highlighting the importance of having high surfactant concentrations in the formulations in order to maximize drug entrapment in the vesicles. Thus, equal mole proportions between the surfactant and cholesterol seems desirable for producing niosomes with appropriate size, size distribution (Table 3) and LTZ entrapment efficiency (Fig. 4A), agreeing with previous observations using conventional niosomes [48]. Interestingly, the increase in the ratio between the non-ionic surfactant and cholesterol beyond 1:1 has decreased the drug entrapment. This might be attributed to that high Tween 80® concentrations have resulted in presence of some surfactant molecules independently in the continuous aqueous phase, increasing the solubility of LTZ in the external aqueous phase at the expense of the niosome bilayers. Alternatively, the presence of high surfactant concentrations may have caused the vesicles to become ‘leaky’, resulting in lower drug entrapment. Amongst the Tween surfactants, Tween 80 in niosome formulations was reported to offer the highest entrapment efficiency [55], justifying its use in this study.

3.4. Influence of storage on LTZ entrapment

Niosome instability during storage is commonly manifested by the increase in particle size and polydispersity owing to vesicle aggregation or fusion; this is accompanied by drug leakage from the vesicles [48]. Size and polydispersity increments are qualitative measures of vesicle stability, and can be associated with limitations such as the range of particle size that can be accurately measured by each instrument and the artifacts associated with every size analysis technique. For this reason, we decided to study the entrapment of LTZ as a quantitative measure for niosome stability; hence the greater the drug leakage (i.e. the lower the entrapment), the lower the formulation stability.

We determined LTZ entrapment after three months of storage at 4 °C or room temperature (RT) and compared these findings to the entrapment determined for freshly prepared niosomes (Fig. 4B). Studies using the traditional separation technique with DW (i.e. apparent entrapment studies) showed that differences in drug entrapment after storage were minimal compared to the freshly prepared vesicles (Fig. 4B). By contrast, the ‘actual entrapment’ studies confirmed that effective separation is important for providing trustable entrapment findings that can be translated into reliable stability studies. Importantly, actual drug entrapment studies revealed that storage of niosomes causes marked leakage of LTZ (i.e. lower actual drug entrapment efficiencies), with more drug losses (i.e. lower entrapment values) at RT compared to 4 °C (Fig. 4B). For example, in F5 formulation, the actual entrapment efficiency decreased from around 74% in the freshly prepared vesicles to less than 20% when the formulation was stored at RT (Fig. 4B). These findings indicate that niosomes should be used as soon as they are prepared, and if storage is necessary, then it should happen at low temperature (e.g. 4 °C). Importantly, the stability study was conducted on niosomes in their aqueous dispersion form not on the proniosomes in their powder form. Our initial investigations showed that, storage of proniosome powders at 4 °C or RT did not affect drug entrapment in accordance to a parallel study we conducted on one these formulations (data not shown). In the future, a broader stability investigation will be implemented to explore more about the storage of proniosome powders.

3.5. Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) was carried out for LTZ-loaded proniosomes versus the pure LTZ powder. LTZ alone showed a sharp endothermic peak corresponding to its melting point with an onset of 185 °C, illustrating the typical crystalline nature of the drug. This peak was persistent even upon processing LTZ in chloroform (in the rotary evaporator) to simulate the process of niosomal preparation (data not shown). Importantly, this distinctive peak of LTZ was absent

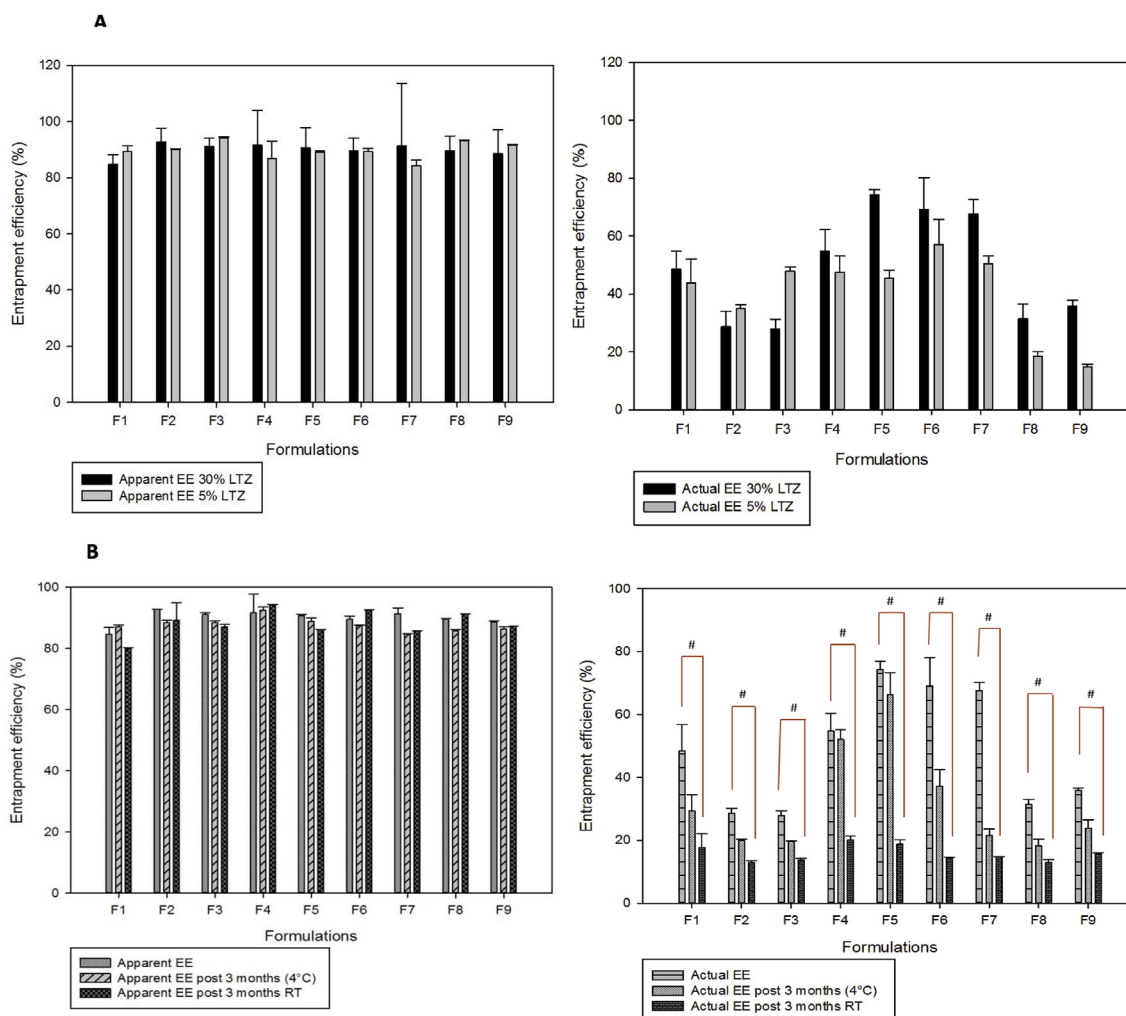


Fig. 4. A. Entrapment efficiency differences when 30% LTZ and 5% LTZ loadings were compared. Data are mean values ($n = 3 \pm \text{SD}$). B. 'Apparent entrapment' and 'actual entrapment' efficiencies during storage at different temperatures. Data are mean values ($n = 3 \pm \text{SD}$).

in all proniosome formulations, regardless of the carbohydrate carrier type (i.e. sucrose or sorbitol) (Fig. 5), indicating conversion of LTZ into its amorphous form. These observations were ascertained further in the subsequent section using XRD. Furthermore, carbohydrate carriers (sorbitol and sucrose) also exhibited sharp melting peaks, indicating that their crystallinity was not affected by coating with lipids such as Tween 80 and cholesterol. It was also noted that different carbohydrate carriers had different melting temperatures, with minimal effect of lipid composition on the melting temperatures of the carbohydrate carriers (Fig. 5).

3.6. X-ray diffraction (XRD) analysis

X-ray diffraction (XRD) was used to evaluate the crystallinity characteristics of LTZ in the proniosome powders. XRD showed that LTZ crystals were detected as sharp and distinctive peaks when evaluated before dissolving the drug in chloroform or even after treating them in the rotary evaporator (simulating the process by which proniosomes were formulated). Hence, using XRD, there was no apparent effect of the organic solvent on the crystallinity characteristics of LTZ. Furthermore, studies on proniosomes showed no distinctive peaks of LTZ in the proniosome formulations, regardless of carbohydrate carrier type (Fig. 6). These findings consolidated our DSC results, where the characteristic peaks of LTZ were absent when it was incorporated into proniosome powders. The current results are in accordance with previous studies that compared the physical state of poorly-water soluble

drugs (e.g. ezetimibe, nateglinide, vinpocetine) incorporated into proniosome formulations [36,56,57]. In our previous investigations using slurry-based proliposomes, we also found that the hydrophobic drug beclomethasone dipropionate becomes amorphous upon incorporation into phospholipid surfactants with CH and using carbohydrate carriers like sorbitol, lactose monohydrate and mannitol [53], indicating that proniosomes and proliposomes can abolish the crystallinity of the incorporated hydrophobic drug.

3.7. Scanning electron microscopy (SEM) studies

Scanning electron microscopy (SEM) images of proniosomes are presented in Fig. 7. It can be observed that surface appearance depends on type of carrier. For instance, the surface of sorbitol particles, prior to coating with lipids, was highly porous and spiky. This was changed into apparently smoother and less spiky upon coating with the lipids, indicating that the porous structure of the carrier was coated successfully with the lipid. By contrast, sucrose particles prior to coating were apparently smooth and non-porous. This was changed upon coating with the lipid, creating what looked to be a flaky surface. For comparison of the surface appearance of proniosomes with that of the thin-film, we also imaged the thin-film using SEM. Thin-films of surfactant, CH and LTZ had a flaky surface that is similar to that of sucrose-based proniosomes. Parallel to this study, we conducted drug content analysis to assess whether the morphology of the carbohydrate carriers (porous surface of sorbitol and flatty surface of sucrose) would affect the

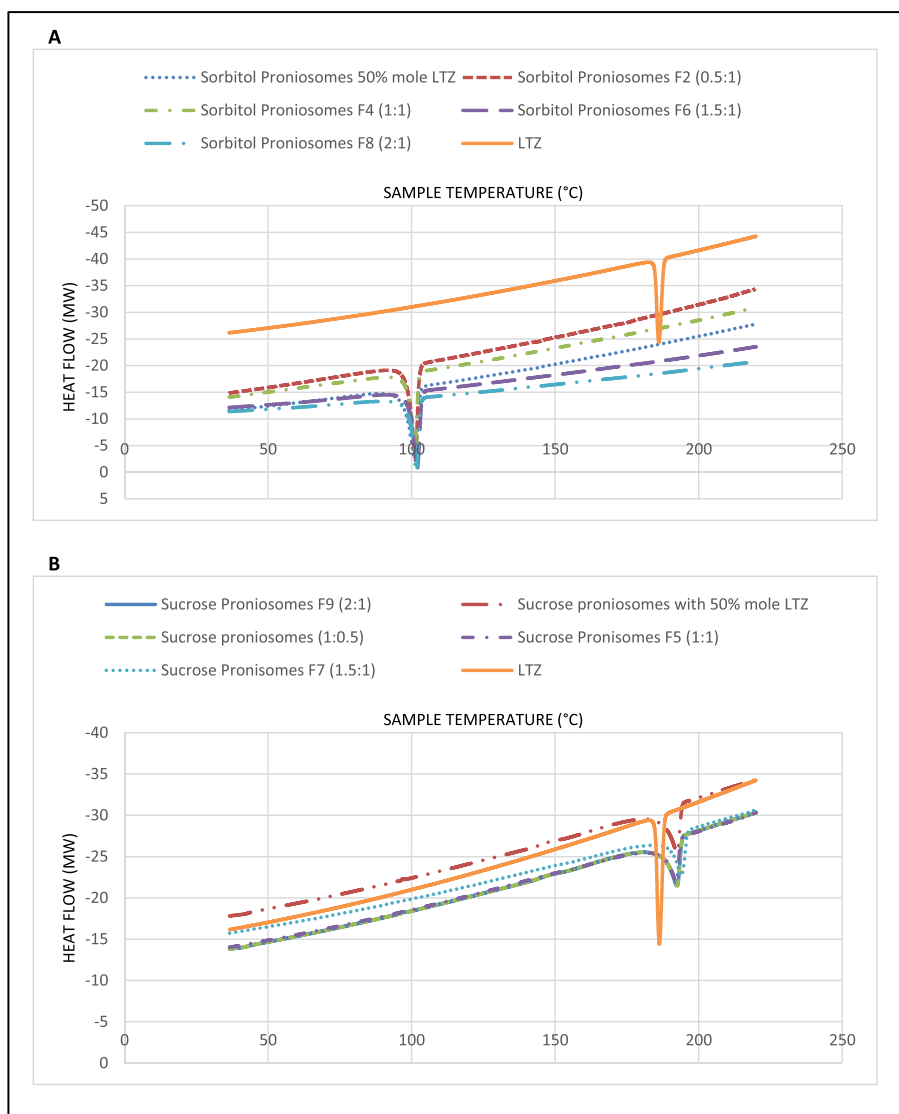


Fig. 5. A. DSC Thermographs of sorbitol-based proniosomes. The absence of LTZ distinctive peak at 185 °C indicates drug conversion into the amorphous form. B. DSC thermographs of sucrose-based proniosomes. The absence of LTZ distinctive peak at 185 °C indicates drug conversion into the amorphous form.

accommodation of LTZ on the carrier surfaces. All proniosome formulations accommodated 95–98% of LTZ originally included (data not shown), indicating that surface morphology had no effect on drug content. Interestingly, the similarity of surface morphology of sucrose-based proniosomes and that of thin-film seemed to end up with niosomes generally having lower PDI values compared to niosomes generated from sorbitol-based proniosomes (Table 3).

3.8. *In vitro* release profile of LTZ

The formulations selected for the release studies were those providing the highest drug entrapment efficiencies and best storage stability; these characteristics were best achieved when CH to surfactant ratio was 1:1. Thus, F4 and F5 were compared against LTZ suspension and F1 (the traditional niosomal formulation made by the thin-film method) (Fig. 8). The drug release profiles were studied over a period of one month, and the mechanism of release was fitted against a number of models, which are zero-order, first-order, Higuchi, Korsmeyer-Peppas, Weibull, Hill equation and Hixon Crowell.

All F1, F4 and F5 formulations were found to have similar release patterns, in which a burst of drug was released without a detectable lag-time in the initial phase over the first 24 h (Fig. 8A). The percentage of

drug release reached approximately $53 \pm 0.5\%$, $65 \pm 1\%$ and $59 \pm 3\%$ for conventional niosomes, sorbitol-based niosomes and sucrose-based niosomes, respectively. These were faster than the release of LTZ from the suspension formulation that required a longer duration of one week to reach the same release levels (Fig. 8B) demonstrated by niosomes over 24 h (Fig. 8A), possibly because the solubility of LTZ was the lowest in the suspension formulation. The drug release was found to happen very slowly after the first 24 h (Fig. 8). Importantly, the cumulative release from niosomes generated from proniosomes was higher than that from the conventional vesicles after 24 h (Fig. 8); however, after a week duration, the release from conventional niosomes reached a very similar level to that of proniosomes-generated niosomes (Fig. 8).

When considering the drug release levels after a month, all formulations have released almost 100% of the originally entrapped LTZ (Fig. 8C). The difference in release patterns between conventional vesicles and those generated from proniosomes is attributed to different formulations. It seems that sugars have affected the initial packing of the niosomal bilayers, contributing to the faster drug release from the proniosome-made formulations (Fig. 8A and B).

Table 4 summarizes the kinetic profiles in r -squared (r^2) values, which were obtained from linear regression analysis for all

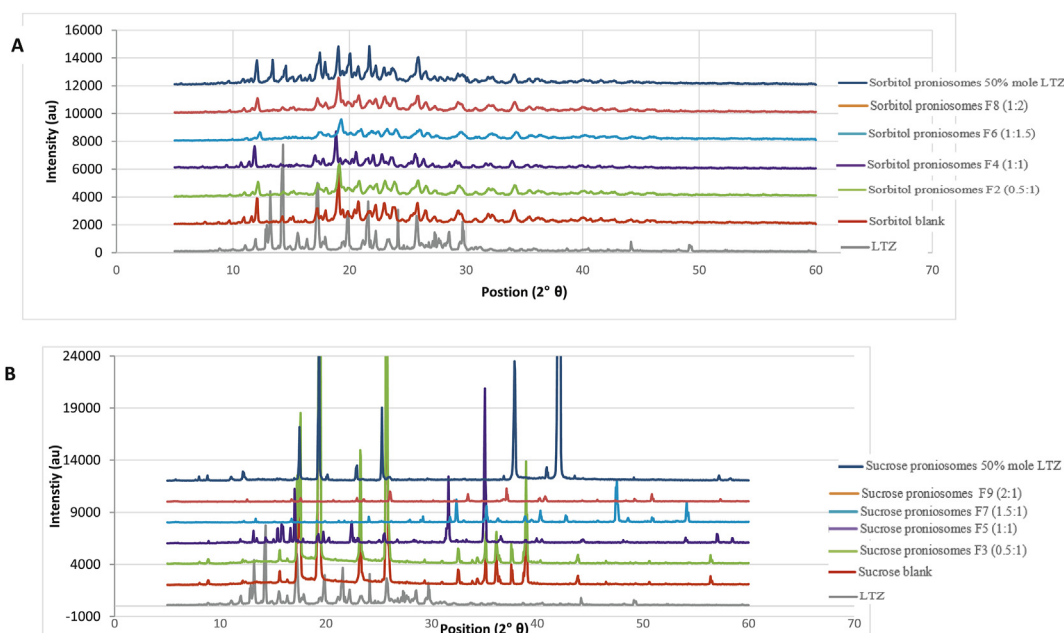


Fig. 6. A. XRD graph for sorbitol based proniosome formulations. B. XRD graph for sucrose based proniosome formulations.

formulations as well as for LTZ suspension as a control comparative formulation. The results showed that proniosomal formulations were consistently best fitted with the Higuchi model, which explains that LTZ diffuses from the vesicles in a slower rate as the distance for diffusion increases. Moreover, it was noted that the formulations followed a biphasic release pattern, in which a surge of LTZ was released into the medium in a fast rate, which was followed by a sustained slower rate of release for the remaining period of investigation (Table 4).

Our present findings are in agreement with those presented by Kamboj et al. (2014) who reported that drug release from niosomes was best fit with Higuchi model followed by Korsmeyer-Peppas model [43]. This confirms that the loaded drug concentration is higher than drug solubility in the niosomal suspension; this provides perfect sink conditions at the niosome's surface. Consequently, the system shows a prolonged release time known as pseudo-steady-state conditions

leading to drug diffusion from niosomes compared to solid suspension. Furthermore, diffusion-based release pattern was reported for zidovudine-loaded niosomal formulations [49]. Biphasic release from niosomes was also reported for ciprofloxacin, with a rapid initial release followed by a slower phase [58].

The fatty acid chain characteristics of the surfactant is influential on drug release from vesicles. The longer the chain, the slower the drug release, which is attributed to the rigidifying effect of long chain surfactants on niosomal membranes [49]. In this study we focused on carbohydrate carrier type and surfactant to CH ratio. Future investigations should explore the effect of type of non-ionic surfactant on physicochemical properties of niosomes generated from proniosomes made using the slurry-based method.

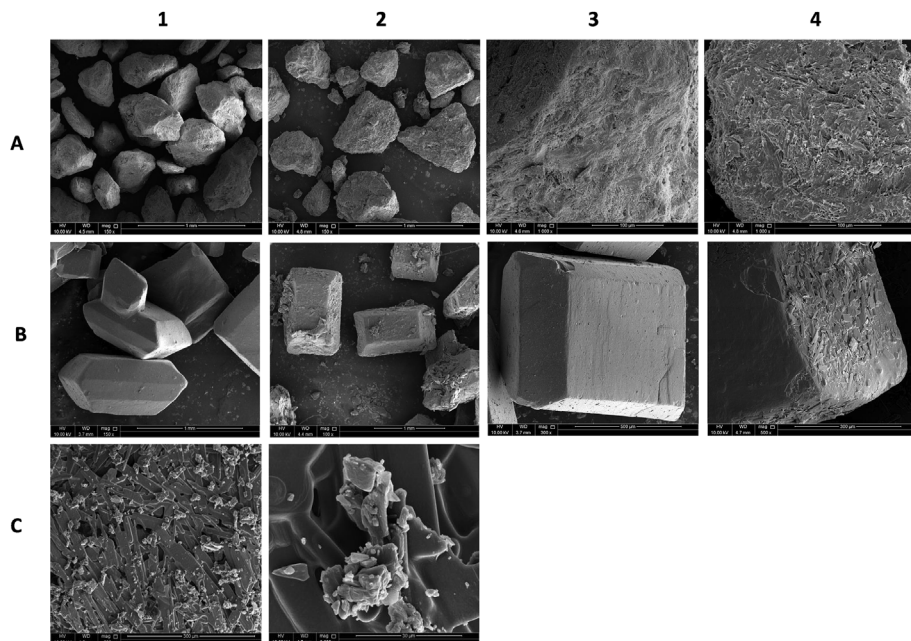


Fig. 7. A. Scanning electron micrographs for sorbitol based proniosomes. (1) Commercial samples of sorbitol demonstrate a surface that looks like crystals that are sharp and rough. (2) Proniosomes prepared using sorbitol, in which the surface is similar but with less well-defined features. (3) and (4) are the same images as (1) and (2), respectively but with higher magnifications. B. Scanning electron microscopy images for sucrose-based proniosomes. (1) Commercial sucrose samples prior to coating with lipid appear smooth and well defined. (2) Proniosomes prepared using sucrose and coated with lipids has a surface that is flaky and rigid. (3) and (4) are the same images as (1) and (2), respectively but with greater magnifications. C. Thin lipid films containing no carbohydrate carriers in two different magnifications.

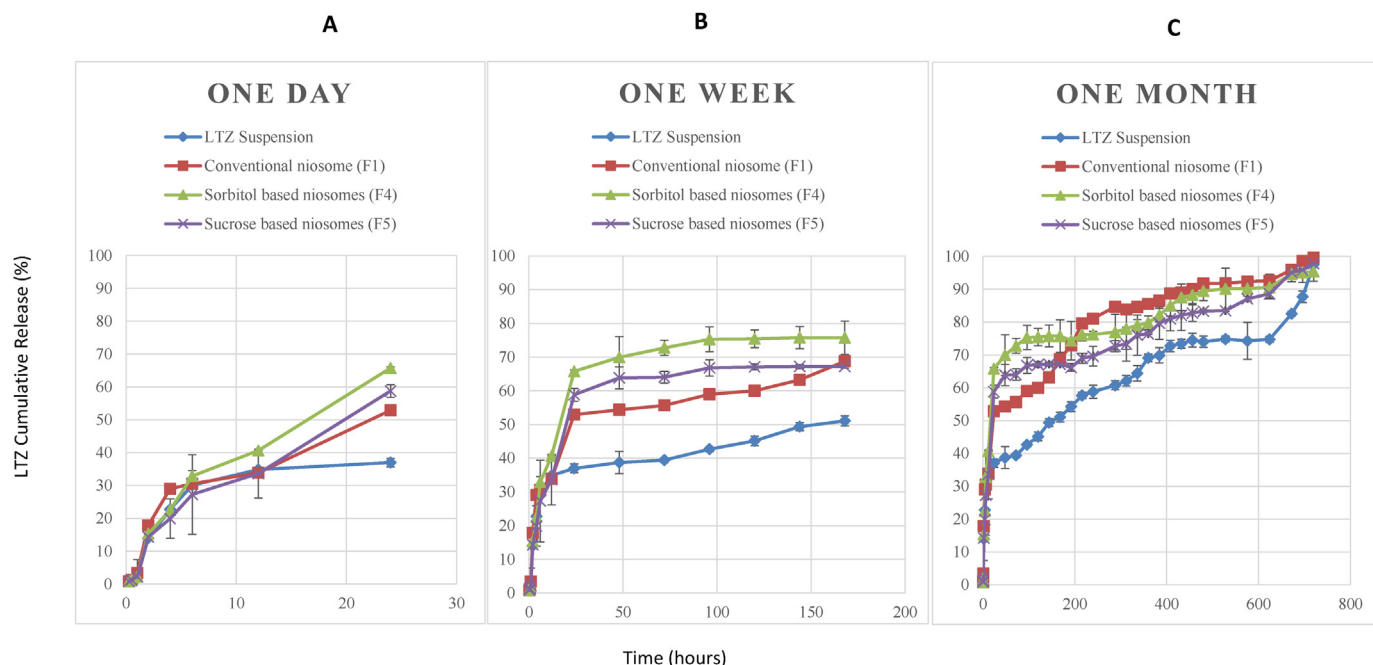


Fig. 8. A. Percentage of LTZ released over a duration of one day for LTZ suspension, Conventional niosomes, Sorbitol-based niosomes and Sucrose-based niosomes ($n = 3 \pm \text{SD}$). B. Percentage of LTZ released over a duration of one week for LTZ suspension, Conventional niosomes, Sorbitol based niosomes and Sucrose based niosomes ($n = 3 \pm \text{SD}$). C. Percentage of LTZ released over a duration of one month for LTZ suspension, Conventional niosomes, Sorbitol based niosomes and Sucrose based niosomes ($n = 3 \pm \text{SD}$).

4. Conclusion

Novel proniosome formulations incorporating the anticancer drug LTZ were designed using sorbitol or sucrose carrier particles, by employing the ethanol slurry method. Niosomes readily generated niosomes upon addition of aqueous phase, and as for conventional niosomes (made using thin film-hydration), the vesicles were reduced in size successfully to the nano range (approx. 100–200 nm) via probe-sonication. Sorbitol-based proniosomes were found to generate niosomes that are slightly larger than conventional niosomes and those generated from sucrose-based proniosomes. All niosomes had negative zeta potential values, regardless of carrier type and lipid composition. Increasing the surfactant concentration made the niosomes smaller in size but did not affect the zeta potential. The lowest polydispersity and highest ‘actual entrapment’ was demonstrated when proniosomes were made with 1:1 CH to surfactant. SEM images showed different surface morphologies of proniosomes in accordance with using different carriers. LTZ is crystalline in its pure powder, as shown by both DSC and XRD; however, it was converted into its amorphous form upon incorporation into proniosomes. Hydration of lipid with deuterium oxide (D_2O) facilitated the separation of entrapped drug, leading to more reliable entrapment efficiency determination, which was referred to as ‘actual entrapment’ which was at highest (approx. 74%) when LTZ was incorporated into sucrose-based proniosomes (CH:T80; 1:1). Storage of

niosomes for 3 months provided evidence of drug leakage, slightly when storage occurred at 4°C and tremendously when formulation was stored at RT. The release study showed that niosomes had a biphasic release profile where the drug was released rapidly during the first 24 h, so that the total amount of drug released reached approx. 65%, 59% and 53% for sorbitol-based proniosomes, sucrose-based proniosomes and conventional niosomes, respectively, whilst the drug in a suspension formulation had less than 40% of the drug released. After one week, the drug release was very slow and similar level of release was exhibited for the three formulations, being approximately 75%, 68% and 69%, respectively, compared to 51% released from the suspension formulation. After one month, all four formulations had already released at least 95% of the drug. The faster release from niosomes compared to the suspension formulation was attributed to the enhanced drug dissolution provided by the surfactant. The slightly faster initial release from proniosome formulations compared to the conventional niosomes is attributed to a possible interaction between the sugars and the polar headgroups of the surfactant, affecting membrane packing and drug release. The release profile from niosomes fits best with the Higuchi model.

CRediT authorship contribution statement

Nada Khudair: Formal analysis, Methodology, Writing - original

Table 4

R-squared (r^2) calculated from linear regression analysis of LTZ release from various kinetic models.

Formulations/Kinetics Model	Zero order	First order	Korsmeyer-Peppas	Higuchi	Hickson-Crowell	Weibull	Hill equation
LTZ Suspension	0.757	0.3526	0.8496	0.9854	0.5066	0.8539	0.2221
F1	0.7602	0.374	0.8539	0.9421	0.5322	0.8858	0.2725
F4	0.5656	0.2937	0.8126	0.9534	0.3915	0.8522	0.2472
F5	0.6892	0.3393	0.8794	0.9661	0.4656	0.8663	0.2385

F1: Conventional niosomes (CH and T80; 1:1).

F4: Sorbitol based niosomes (CH and T80; 1:1).

F5: Sucrose based niosomes (CH and T80; 1:1).

draft. **Abdelali Agouni**: Formal analysis, Writing - review & editing. **Mohamed A. Elrayess**: Formal analysis, Writing - review & editing. **Mohammad Najlah**: Formal analysis, Writing - review & editing. **Husam M. Younes**: Data curation, Formal analysis, Writing - original draft, Supervision. **Abdelbary Elhissi**: Data curation, Formal analysis, Writing - original draft, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jddst.2020.101721>.

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Contribution statement

Mrs. Nada Khudair was the MSc student. She performed the experiments, analyzed the results and wrote the first draft of the paper. Dr. Abdelali Agouni, Dr. Mohamed Elrayess, and Dr. Mohammad Najlah contributed to the experimental design, data analysis and manuscript review. Dr. Husam M. Younes and Dr. Abdelbary Elhissi contributed by, interpreting and analyzing the data, writing the manuscript and supervising the student and guiding her work. All authors approved the final version of the manuscript.

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